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# Arachidonoyl-Coenzyme A Synthetase and Nonspecific Acyl-Coenzyme A Synthetase Activities in Purified Rat Brain Microvessels

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**Abstract:** Purified rat brain microvessels were prepared to demonstrate the occurrence of acyl-CoA (EC 6.2.1.3) synthesis activity in the microvasculature of rat brain. Both arachidonoyl-CoA and palmitoyl-CoA synthesis activities showed an absolute requirement for ATP and CoA. This activity was strongly enhanced by magnesium chloride and inhibited by EDTA. The apparent $K_m$ values for acyl-CoA synthesis by purified rat brain microvessels were 4.0 $\mu M$ and 5.8 $\mu M$ for palmitic acid and arachidonic acid, respectively. The apparent $V_{max}$ values were 1.0 and 1.5 nmol $\cdot$ min$^{-1}$ $\cdot$ mg protein$^{-1}$ for palmitic acid and arachidonic acid, respectively. Cross-competition experiments showed inhibition of radiolabelled arachidonoyl-CoA formation by 15 $\mu M$ unlabelled arachidonic acid, with a $K_i$ of 7.1 $\mu M$, as well as by unlabelled docosahexaenoic acid, with a $K_i$ of 8.0 $\mu M$. Unlabelled palmitic acid and arachidic acid had no inhibitory effect on arachidonoyl-CoA synthesis. In comparison, radiolabelled palmitoyl-CoA formation was inhibited competitively by 15 $\mu M$ unlabelled palmitic acid, with a $K_i$ of 5.0 $\mu M$ and to a much lesser extent by arachidonic acid ($K_i$, 23 $\mu M$). The $V_{max}$ of palmitoyl-CoA formation obtained on incubation in the presence of the latter fatty acids was not changed. Unlabelled arachidic acid and docosahexaenoic acid had no inhibitory effect on palmitoyl-CoA synthesis. Both arachidonoyl-CoA and palmitoyl-CoA synthesis activities were thermolabile. Arachidonoyl-CoA formation was inhibited by 75% after 7 min at 40°C whereas a 3-min heating treatment was sufficient to produce the same relative inhibition of palmitoyl-CoA synthesis. These data together strongly suggest that rat brain microvessels have the capacity to catalyze specifically the formation of acyl-CoA derivatives from several polyunsaturated long-chain fatty acids, including arachidonic acid in the first place. Besides this particular arachidonoyl-CoA synthetase, palmitic acid could be activated with the aid of a second acyl-CoA synthetase. **Key Words:** Arachidonic acid—Acyl-CoA synthetase—Brain—Microvessels. **Morand O. et al.** Arachidonoyl-coenzyme A synthetase and nonspecific acyl-coenzyme A synthetase activities in purified rat brain microvessels. *J. Neurochem.* **48,** 1150–1156 (1987).

Brain microvessel endothelium cells constitute the major component of the so-called blood–brain barrier (Oldendorf, 1977). This continuous layer is formed of cells joined together by tight junctions that do not allow intercellular solute diffusion. Hexoses, amino acids, purine compounds, monocarboxylic acids, as well as several other physiological compounds are transported across the blood–brain barrier with the aid of carrier-mediated mechanisms (Pardridge and Oldendorf, 1977). Several studies were designed to clarify the relationships between brain microvessel lipid synthesis and degradation and the functional role of this metabolism. Hence, one can assume that this very specific structure might play a role in (1) uptake and hydrolysis of serum lipid units and subunits, (2) providing energy from $\beta$-oxidation of fatty acids, (3) synthesis of new lipids, (4) synthesis of prostaglandins as well as leukotrienes, and (5) providing lipids and fatty acids to the neural tissue.

Exogenous radiolabelled fatty acids injected to animals are transported across the blood–brain barrier and further incorporated into lipids of brain cells and myelin (Dhopeshwarkar and Mead, 1973; Morand et al., 1981). Both lipoprotein lipase and acid lipase activities were detected in rabbit brain microvessels, suggesting that lipoproteins are metabolized within the

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*Abbreviations used:* DMSO, dimethyl sulfoxide; DTT, dithiothreitol.

cerebral vasculature (Brecher and Kuan, 1979). Diacylglycerol lipase and kinase activities were measured in rat brain microvessel preparations (Hee-Cheong et al., 1985). Fatty acid oxidation might be of particular importance to brain microvessels because optimal ion transport into isolated cerebral microvessels can be obtained only if palmitic acid is present in addition to glucose (Goldstein, 1979). Bovine microvessels produce similar amounts of carbon dioxide from glucose and palmitate (Betz and Goldstein, 1981). Radioactive phospholipids and neutral lipids are readily synthesized by isolated rat brain microvessels incubated in the presence of [1-$^{14}$C]acetate (Homayoun et al., 1985). Furthermore, fatty acid oxidation activity was shown to be much higher in brain microvessels than in other vessels and was decreased in some pathological conditions such as hypertension, aging, and diabetes mellitus (Morisaki et al., 1982, 1983). In rat cerebral microvessels, fatty acids might be also diverted to prostaglandin synthesis by a cyclooxygenase (Gerritsen et al., 1980) as well as to leukotriene synthesis by a lipoxygenase (Baba et al., 1985). Interestingly, Spector et al. (1983) suggested that the amount of free arachidonic acid of the endothelial cells might modulate the capacity of the endothelium to produce prostaglandins. It is clear that arachidonoyl-CoA formation can divert free arachidonic acid away from the latter pathway. More generally, enzymatic acyl-CoA (EC 6.2.1.3) formation is known to be nearly an obligatory step prior to fatty acid oxidation and esterification into phospholipids and neutral lipids. In various tissues and in particular in brain, this enzymatic activity is dependent on the length and the degree of unsaturation of the acyl chain (Murphy and Spence, 1980, 1982). Reddy and Bazan (1983) showed that rat brain microsomes express a specific arachidonoyl-CoA synthesis activity and hypothesized that this activity might be involved in limiting eicosanoid formation and also participate in the retention of essential fatty acids in the CNS. Similar observations were made by Wilson et al. (1982) using human platelets. On the contrary, Morisaki et al. (1986) showed that the acyl-CoA synthetase of the rat glomeruli has a unique broad specificity for fatty acids and that there is no arachidonic acid-specific ligase in this kidney subfraction.

This work was performed to investigate the activity of acyl-CoA formation by purified rat brain microvessels. Palmitic acid and arachidonic acid were tested as samples of respectively saturated and polyunsaturated fatty acid series regarding the specificity of the enzyme(s) producing CoA-activated acyl chains. Fatty acid cross-competition experiments as well as heat-denaturation assays were performed and suggest that purified rat brain microvessels can activate rather specifically polyunsaturated fatty acids. Kinetic parameters were determined and compared to the acyl-CoA synthesis activities measured in total brain homogenates.

## MATERIALS AND METHODS

### Materials

Dextran (MW 70,000) was obtained from Pharmacia, nylon mesh (118 μm) from Desjobert (Paris, France), and glass beads from B-Braun (Mensugen, F.R.G.). [9,10(n)-$^3$H]Palmitic acid (40 Ci/mmol) and [5,6,8,9,11,12,14,15(n)-$^3$H]arachidonic acid (87 Ci/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.) and ACSII scintillation liquid from Amersham (U.K.). Unlabelled palmitic acid (C16:0), arachidic acid (C20:0), arachidonic acid [C20:4(n-6)], and 4,7,10,13,16,19-docosahexaenoic acid [C22:6(n-3)]; ATP; CoA; dithiothreitol (DTT); Triton X-100; and albumin (bovine, fatty acid-free, fraction V) were obtained from Sigma (St. Louis, MO, U.S.A.). Dimethylsulfoxide (DMSO), isopropanol, and *n*-heptane were provided by Merck.

### Preparation of purified rat brain microvessels

Purified rat brain microvessels were prepared according to the method described by Goldstein et al. (1975) and slightly modified as follows. Fifteen Sprague-Dawley rats (2 months old) were killed by decapitation. The brains were immediately removed and placed in cold buffer made of oxygen-saturated Ringer solution containing 1.2 m*M* $MgCl_2$, 15 m*M* *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.4, and 1% (wt/vol) fraction V bovine serum albumin. The brainstem, cerebellum, and meninges were discarded. Cortical hemispheres free of choroid plexus and ependyma were minced with scissors in buffer. The tissue was homogenized in a glass homogenizer with a Teflon pestle (0.25-mm clearance) at 390 rpm (20 strokes). The homogenate was centrifuged at 1,000 *g* for 10 min. The pellet was resuspended in cold buffer containing 17.5% (wt/vol) Dextran to a concentration of 1 g of fresh tissue per 13 ml of the Dextran solution and centrifuged at 4,000 *g* for 15 min. The new pellet, which consisted of free nuclei and microvessels, was resuspended in buffer and then passed through a 118-μm nylon mesh under gentle vacuum. The microvessels were separated from nuclei by passing the suspension through a 1.2 × 1.5 cm column containing 0.25-mm diameter glass beads. Nuclei were removed by extensive washing with buffer whereas microvessels remained attached to the glass beads. After resuspension in buffer, microvessels were collected by gentle agitation and subsequent sedimentation of the beads. The microvessel suspension was centrifuged at 500 *g* for 5 min. To remove traces of albumin the pellet was washed twice with a solution made of 155 m*M* KCl and 5 m*M* $NaH_2PO_4$, pH 7.4. The quality of the preparation was assessed by its appearance in phase contrast microscopy. Purified brain microvessels were stored in a small volume of potassium chloride buffer at −30°C for no more than 4 weeks or immediately incubated with fatty acids and assayed for acyl-CoA synthesis activity as described below.

### Incubation of brain microvessel and total brain homogenates with radiolabelled fatty acids

Purified rat brain microvessels in a pellet were resuspended in cold standard buffer containing 155 m*M* $KCl_2$, 5 m*M* $NaH_2O_4$, and 2 m*M* $MgCl_2$ (pH 7.4). Eventually, magnesium chloride was omitted when testing its requirement in the enzymatic reaction. Microvessels were homogenized in a glass homogenizer with a Teflon pestle driven at 400 rpm (20 strokes). Rat forebrains were homogenized in cold

standard buffer under similar conditions. Homogenates were diluted to a given protein concentration prior to the final dilution initiating the incubation.

[$^3$H]Palmitic acid and [$^3$H]arachidonic acid were diluted each with either nonradioactive palmitic acid or arachidonic acid to a final specific radioactivity of 6.5 and 4.5 mCi/mmol, respectively, and solubilized in DMSO to a concentration of 1 $\mu$mol/ml. Radiolabelled fatty acid in DMSO was mixed at a concentration of 50 $\mu M$ with the standard buffer containing Triton X-100. In a glass tube, varying volumes of the radiolabelled fatty acid solution were completed with buffer containing Triton X-100 to attain a volume of 125 $\mu$l and mixed with 250 $\mu$l of buffer containing ATP, CoA, and DTT. For assaying acyl-CoA synthesis, brain microvessel or total brain homogenate was added in a volume of 125 $\mu$l and further incubated at 37°C. Final conditions were: 0.1 mg tissue protein/ml, 1 m$M$ ATP, 40 $\mu M$ CoA, 40 $\mu M$ DTT, 0.1% Triton X-100 (wt/vol), and 0.4–10 $\mu M$ radiolabelled fatty acid in a volume of 0.5 ml. For cross-inhibition experiments, unlabelled fatty acids were diluted in DMSO and mixed with the solution already containing radiolabelled fatty acid and Triton X-100. The DMSO, the final concentration of which did not exceed 1.5% (vol/vol), had no adverse effect on the enzymatic reaction tested.

### Assay of acyl-CoA synthesis activity

When assaying the synthesis of acyl-CoA incubations were performed as described above. For stopping the incubation, 0.5 ml of incubation mixture received 2 ml of a Dole mixture: isopropanol/heptane/2 $M$ sulfuric acid (40:10:2, by vol) (Dole, 1956) and treated as follows. A 0.7-ml volume of water was added as well as 1.5 ml of heptane containing 5 mg/ml of nonradioactive palmitic acid as a carrier for unreacted radiolabelled fatty acids. The mixture was vortex-mixed vigorously. The upper phase was removed and the lower phase was extracted thrice more with 2 ml each of heptane containing nonradioactive palmitic acid (Banis and Tove, 1974; Reddy and Bazan, 1983). The four upper phases were pooled in a scintillation vial and counted. The tube was finally washed once with 0.5 ml DMSO to remove all remaining radioactivity and this fraction was counted separately. The addition of the counts of the lower phase and the DMSO fraction represented the radioactivity of activated fatty acids. Background values were determined by incubating in the absence of membranes or at zero time. All values were calculated from two duplicate experiments: variations did not exceed 5–10% of each value.

### Heat inactivation of acyl-CoA synthesis

Samples of brain microvessel homogenates (2 mg protein/ml) were heated at 40°C in separate tubes. At various times, cold buffer was added to each sample which was subsequently assayed for [$^3$H]arachidonoyl-CoA and [$^3$H]palmitoyl-CoA formation at 37°C and for 5 min under standard conditions. In this set of experiments, [$^3$H]arachidonoyl-CoA synthesis was determined in the presence of 15 $\mu M$ unlabelled palmitic acid.

### Radioactivity and protein content determinations

All radioactive samples were counted in 10 ml of ACSII scintillation liquid in a 460CD Packard spectrometer with automatic external standard quenching correction. The protein content was determined by means of a fluorescence procedure (Böhlen et al., 1973).

## RESULTS

### General observations

Purified rat brain microvessels incubated in the presence of 6 $\mu M$ radiolabelled palmitic acid, 40 $\mu M$ CoA, 1 m$M$ ATP, 40 $\mu M$ DTT, 2 m$M$ $MgCl_2$, and 0.1% Triton X-100 (wt/vol) formed a reaction product that was extracted in the lower phase of the Dole solvent partition system. The amount of reaction product increased almost linearly for the first 10 min, reaching 1.5 nmol/mg protein, and slowed down further to 4.0 nmol/mg protein at 30 min (data not shown in a figure). Increasing microvessel concentrations (from 25 to 200 $\mu$g protein/ml) in the presence of radiolabelled palmitic acid or arachidonic acid resulted in a linear increase of reaction product synthesis. When total brain homogenate was used as a source of enzyme, a linear increase of the amount of reaction product was also observed in the same range of protein concentration and for at least 10 min. According to these data, all subsequent incubations were performed in the presence of 0.1 mg microvessel protein/ml and for 5 min.

The requirements for various substrates and cofactors are shown in Table 1. The omission of ATP or CoA caused a 70–100% decrease in activity, suggesting their absolute requirement for the formation of the reaction product. The absence of exogenous $MgCl_2$ decreased the activity by 85–90%. Incubation in the presence of 10 m$M$ EDTA and absence of $MgCl_2$ was followed by a nearly complete inhibition of the activity. The omission of DTT caused a 50% decrease in arachidonoyl-CoA synthesis activity and had no effect on palmitoyl-CoA synthesis. The lower phase of the Dole partition system may also contain radiolabelled fatty acid linked to phospholipids. However, acyl-CoA formation from fatty acid and CoA in the presence of ATP is an obligatory metabolic step preceding phospholipid esterification. Consequently, the entire reaction product extracted in the lower phase was defined as acyl-CoA.

**TABLE 1.** *Substrate requirement for acyl-CoA synthesis by purified rat brain microvessels*

| | [$^3$H]Palmitic acid | [$^3$H]Arachidonic acid |
|---|---|---|
| Complete system | 100 | 100 |
| − ATP | 0 | 7 |
| − CoA | 25 | 27 |
| − $MgCl_2$ | 9 | 15 |
| − $MgCl_2$ + EDTA (10 m$M$) | 0 | 7 |
| − DTT | 97 | 51 |

Brain microvessels were incubated with 10 $\mu M$ [$^3$H]palmitic acid or 10 $\mu M$ [$^3$H]arachidonic acid in a complete system or in the absence of one of the cosubstrates or cofactors. The complete system contained 40 $\mu M$ CoA, 1 m$M$ ATP, 40 $\mu M$ DTT, 2 m$M$ $MgCl_2$, and 0.1% Triton X-100 in standard buffer. Data are expressed as percentages of the control value (i.e., the complete system).

### Kinetic properties of acyl-CoA synthesis

Increasing concentrations of radiolabelled palmitic acid or arachidonic acid were added to microvessel homogenate and acyl-CoA synthesis was measured after 5-min incubation at 37°C. A Michaelis-type hyperbolic relationship was observed between increasing fatty acid concentrations and acyl-CoA synthesis (data not shown in a figure). Replotting arachidonoyl-CoA synthesis in the double-reciprocal Lineweaver-Burk presentation permitted calculation of an apparent $K_m$ of 5.8 $\mu M$ and an apparent $V_{max}$ of 1.5 nmol·min$^{-1}$·mg protein$^{-1}$ (Fig. 1; Table 2). Replotting palmitoyl-CoA synthesis permitted calculation of an apparent $K_m$ of 4.0 $\mu M$ and an apparent $V_{max}$ of 1.0 nmol·min$^{-1}$·mg protein$^{-1}$ (Fig. 2; Table 2). In comparison, similar experiments were performed using total forebrain homogenate as a source of enzyme and kinetic parameters were calculated. Palmitoyl-CoA and arachidonoyl-CoA synthesis by forebrain homogenate occurred with $K_m$ values of 5.5 and 5.1 $\mu M$, and $V_{max}$ values of 1.3 and 1.3 nmol·min$^{-1}$·mg protein$^{-1}$, respectively.

### Substrate cross-competition experiments

Competition experiments were performed to investigate the substrate specificity of acyl-CoA synthesis when assayed in purified rat brain microvessel homogenate. Microvessel homogenates were incubated for 5 min at 37°C with increasing concentrations of radiolabelled palmitic acid or arachidonic acid and in the presence of 15 $\mu M$ each of nonradioactive palmitic acid, arachidic acid, arachidonic acid, and docosahexaenoic acid tested as possible inhibitors of palmitoyl-CoA or arachidonoyl-CoA synthesis. Unlabelled arachidonic acid competitively inhibited the formation of [$^3$H]arachidonoyl-CoA, giving a $K_i$ of 7.1 $\mu M$ (Fig. 1; Table 2). Docosahexaenoic acid also produced competitive inhibition of [$^3$H]arachidonoyl-CoA synthesis, with a $K_i$ of 8 $\mu M$. In contrast, palmitic acid as well as arachidic acid did not inhibit the synthesis of [$^3$H]arachidonoyl-CoA and rather caused a slight de-

**TABLE 2.** *Kinetic parameters of palmitoyl-CoA and arachidonoyl-CoA synthesis by purified rat brain microvessels calculated on cross-inhibition assays with various saturated and polyunsaturated fatty acids*

| Substrate(s) | $K_m$ ($\mu M$) | $K_i$ ($\mu M$) | $V_{max}$ (nmol·min$^{-1}$·mg protein$^{-1}$) |
|---|---|---|---|
| Arachidonoyl-CoA synthesis | | | |
| [$^3$H]C20:4(n-6) | 5.8 | — | 1.5 |
| [$^3$H]C20:4(n-6) + C20:4(n-6) | 18.0 | 7.1 | 1.5 |
| [$^3$H]C20:4(n-6) + C16:0 | 4.0 | NC | 1.1 |
| [$^3$H]C20:4(n-6) + C20:0 | 3.8 | NC | 2.0 |
| [$^3$H]C20:4(n-6) + C22:6(n-3) | 16.6 | 8.0 | 2.5 |
| Palmitoyl-CoA synthesis | | | |
| [$^3$H]C16:0 | 4.0 | — | 1.0 |
| [$^3$H]C16:0 + C16:0 | 16.0 | 5.0 | 1.0 |
| [$^3$H]C16:0 + C20:0 | 3.3 | NC | 1.2 |
| [$^3$H]C16:0 + C20:4(n-6) | 6.6 | 23.0 | 1.0 |
| [$^3$H]C16:0 + C22:6(n-3) | 3.1 | NC | 0.7 |

See detailed experimental procedure in the legends to Figs. 1 and 2. $K_m$ values relate to radiolabelled arachidonic acid and palmitic acid, and $K_i$ values to the unlabelled fatty acids tested as potential inhibitors. NC, not calculated.

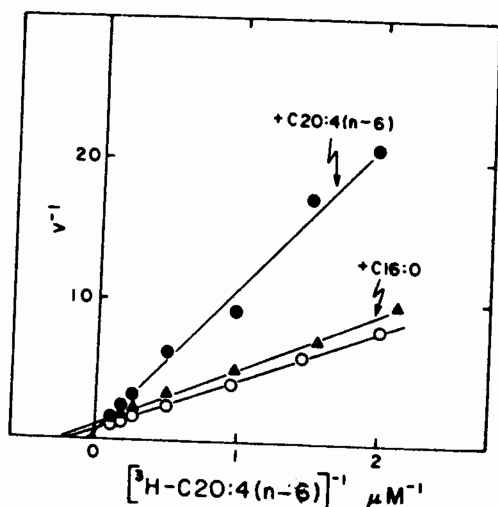


**FIG. 1.** Inhibition of arachidonoyl-CoA synthetase activity by various unlabelled fatty acids. Brain microvessels were incubated for 5 min at 37°C with increasing concentrations of [$^3$H]arachidonic acid in the presence each of 15 $\mu M$ unlabelled palmitic acid (▲ — ▲) or arachidonic acid (● — ●) or in the absence of the additional unlabelled fatty acid (○ — ○). Standard buffer contained 40 $\mu M$ CoA, 1 mM ATP, 40 $\mu M$ DTT, 2 mM $MgCl_2$, and 0.1% Triton X-100. The amount of radiolabelled arachidonoyl-CoA produced was determined and expressed as nmol·min$^{-1}$·mg protein$^{-1}$.

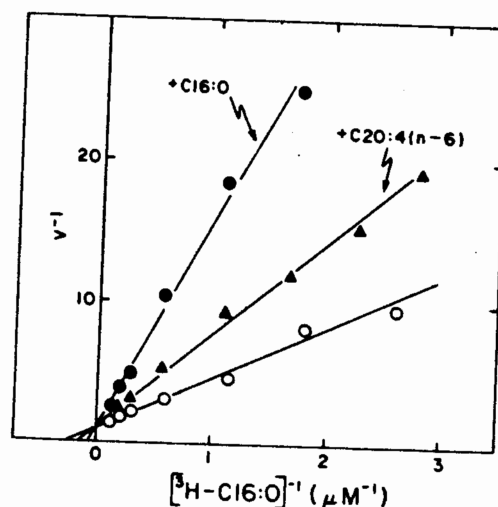


**FIG. 2.** Inhibition of palmitoyl-CoA synthetase activity by various unlabelled fatty acids. Brain microvessels were incubated for 5 min at 37°C with increasing concentrations of [$^3$H]palmitic acid in the presence each of 15 $\mu M$ unlabelled palmitic acid (● — ●) or arachidonic acid (▲ — ▲) or in the absence of additional unlabelled fatty acid (○ — ○). Standard buffer contained 40 $\mu M$ CoA, 1 mM ATP, 40 $\mu M$ DTT, 2 mM $MgCl_2$, and 0.1% Triton X-100. The amount of radiolabelled palmitoyl-CoA produced was determined and expressed as nmol·min$^{-1}$·mg protein$^{-1}$.

crease of the $K_m$ value of radiolabelled arachidonic acid. The $V_{max}$ of [$^3$H]arachidonoyl-CoA synthesis obtained in the presence of these various unlabelled fatty acids remained almost unchanged. Further, the conversion of [$^3$H]palmitic acid into [$^3$H]palmitoyl-CoA was measured in the presence of unlabelled fatty acids (Fig. 2; Table 2). [$^3$H]Palmitoyl-CoA formation was inhibited by palmitic acid and slightly by arachidonic acid, giving $K_i$ values of respectively 5.0 $\mu M$ and 23.0 $\mu M$. Arachidic acid and docosahexaenoic acid did not inhibit [$^3$H]palmitoyl-CoA formation and actually caused a decrease of the $K_m$ value for radiolabelled palmitic acid. The $V_{max}$ of [$^3$H]palmitoyl-CoA formation obtained on incubation in the presence of these unlabelled fatty acids was not affected.

**Effect of heat inactivation of acyl-CoA synthesis**

Brain microvessel homogenates were heated at 40°C for various times and subsequently assayed for palmitoyl-CoA and arachidonoyl-CoA synthesis activities. As shown in Fig. 3, heat denaturation of the synthetase system follows different kinetics depending on whether palmitic acid or arachidonic acid was employed as precursor. [$^3$H]Arachidonoyl-CoA formation was inhibited by 75% after 7 min at 40°C whereas a 3-min heat treatment was sufficient to produce the same relative inhibition of [$^3$H]palmitoyl-CoA synthesis. In the experiment shown, [$^3$H]arachidonoyl-CoA synthesis was assayed in the presence of 15 $\mu M$ unlabelled palmitic acid to eliminate the probable capability of the palmitoyl-CoA synthetase to activate arachidonic acid.

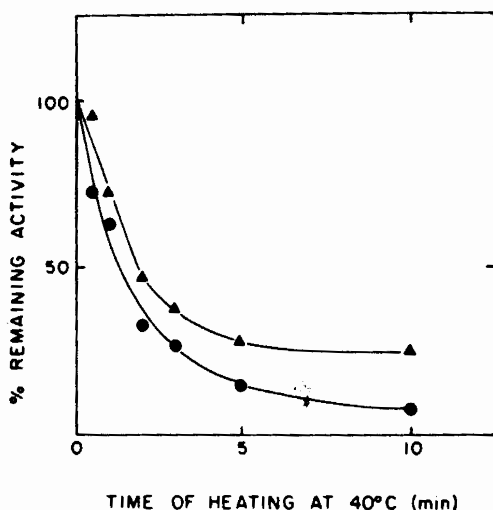


**FIG. 3.** Heat inactivation of arachidonoyl-CoA and palmitoyl-CoA synthesis by brain microvessel homogenates. Brain microvessel homogenates were heated at 40°C for various times and assayed for palmitoyl-CoA (● — ●) and arachidonoyl-CoA (▲ — ▲) synthesis. Arachidonoyl-CoA synthesis was assayed in the presence of 15 $\mu M$ unlabelled palmitic acid. Results are expressed as the percentage of control activity (unheated microvessel homogenates).

## DISCUSSION

This article provides the first description of two distinct acyl-CoA synthesis activities in purified rat brain microvessels. Both radiolabelled palmitic acid and arachidonic acid could be converted to palmitoyl-CoA and arachidonoyl-CoA, respectively. This reaction showed absolute requirement for ATP, CoA, and $MgCl_2$. On omission of CoA, residual activity was observed, suggesting that endogenous CoA might be used during incubation or that activation is unlikely to occur in the absence of that particular cosubstrate. Reddy and Bazan (1983) indicated that conversion of arachidonic acid to arachidonoyl-CoA in rat brain microsomes was very rapid and showed linearity only for the first 20 s of incubation. On the other hand, Murphy and Spence (1980) assayed long-chain fatty acid: CoA ligase in rat brain homogenate and subcellular fractions under 5-min incubation tests. In our work, palmitoyl-CoA synthesis activity was linear for about 10 min. Incubation conditions including detergent and cosubstrate concentrations, the type of fatty acid used as substrate, as well as the type of tissue tested might account for these discrepancies. In this respect, purified rat brain microvessels do represent a very particular structure of the CNS tissue and should be considered as such.

For comparison, kinetic parameters of acyl-CoA synthesis activity were determined in brain microvessel and forebrain homogenates. Palmitoyl-CoA and arachidonoyl-CoA formation by these fractions was saturable when increasing substrate concentration. No striking differences were observed between apparent $K_m$ for palmitic acid in both microvessel and forebrain homogenates. Apparent $K_m$ values varied within 4.0–5.8 $\mu M$ and were two- to eightfold lower than those reported by Reddy and Bazan (1983) for arachidonic acid in rat brain microsomes and by Murphy and Spence (1980) for oleic acid in rat brain microsomes and homogenate. The presence of endogenous unlabelled fatty acids cannot be entirely ruled out and might account for a substantial isotopic dilution of the radiolabelled substrate added, leading to the underestimation of $K_m$ values. However, Cenedella et al. (1975) indicated that endogenous fatty acids are probably present at low concentration (0.3–0.8 $\mu$mol/g of brain tissue). This feature is certainly less relevant in the case of brain microvessels which were prepared and purified in the presence of 1% albumin. The latter provides an effective acceptor for fatty acids released in the CNS tissue on decapitation. Consequently, a double-label assay system using both radiolabelled CoA and radiolabelled fatty acids was not employed in this work.

Further examination of acyl-CoA synthesis activities in rat brain microvessel homogenates involved cross-competition experiments using radiolabelled palmitic acid and radiolabelled arachidonic acid as well as other nonradioactive saturated and polyunsat-

urated fatty acids. Therefore, $K_i$ values of these putative inhibitors were calculated using a derivation of Michaelis equation as follows: $K_i = K_m (K'_m - K_m)^{-1}$ [I]. Accordingly, unlabelled arachidonic acid competitively inhibited the formation of [$^3$H]arachidonoyl-CoA, with a $K_i$ of 7.1 $\mu M$ and unchanged $V_{max}$. Unlabelled docosahexaenoic acid produced similar competitive inhibition of [$^3$H]arachidonoyl-CoA synthesis, with a $K_i$ of 8 $\mu M$. Both $K_i$ values were relatively close to the $K_m$ for [$^3$H]arachidonic acid, suggesting that this enzyme might recognize several polyunsaturated fatty acids as active substrates. In contrast, palmitic acid did not inhibit the formation of [$^3$H]arachidonoyl-CoA. Saturated arachidic acid has the same chain length as arachidonic acid but remains a poor competitor of arachidonoyl-CoA synthesis. These data, summarized in Table 2, strongly support the occurrence of polyunsaturated fatty acid-specific acyl-CoA synthetase in purified rat brain microvessels.

Palmitoyl-CoA synthesis was competitively inhibited by nonradioactive palmitic acid as well as by arachidonic acid (Fig. 2). The $K_i$ of nonradioactive palmitic acid as inhibitor of [$^3$H]palmitoyl-CoA synthesis was 5.0 $\mu M$, i.e., nearly equal to the $K_m$ for [$^3$H]palmitic acid (4.0 $\mu M$). On the contrary, nonradioactive arachidonic acid was but a poor inhibitor of [$^3$H]palmitoyl-CoA synthesis, exhibiting a higher $K_i$ value (23 $\mu M$). Thus, the $K_m$ for [$^3$H]arachidonic acid was strongly different from the $K_i$ of arachidonic acid as inhibitor of [$^3$H]palmitoyl-CoA synthesis. These data together suggest that two acyl-CoA synthetases occur in purified rat brain microvessels. One activity exhibits its specificity for arachidonic acid and very likely for some other polyunsaturated fatty acids. Since arachidic acid, arachidonic acid, and docosahexaenoic acid had actually no or little effect on palmitoyl-CoA formation one may conclude that this second acyl-CoA synthetase is rather specific toward palmitic acid. However, a final conclusion would be reached once many other fatty acids are tested. As proposed by Wilson et al. (1982), a polyunsaturated fatty acid-specific ligase may control the level of free arachidonic acid by diverting arachidonic acid away from cyclooxygenase and into membrane phospholipids. A second activity might account for the formation of other acyl-CoA derivatives preceding their incorporation into cellular neutral lipids and phospholipids as well as $\beta$-oxidation in the mitochondria. According to Goldstein (1979), the latter process might be of utmost importance for brain microvessels, supporting the quantitative and qualitative regulatory functions of the so-called blood–brain barrier.

Both palmitoyl-CoA and arachidonoyl-CoA synthesis activities were notably affected on heating at 40°C, suggesting that these enzymes might be rather unstable. Interestingly, the CoA activation of [$^3$H]palmitic acid was more rapidly inactivated than that of [$^3$H]arachidonicic acid (Fig. 3). Despite opposite findings previously shown in platelets by Wilson et al. (1982), it remains clear that this difference in the time course of heat inactivation of palmitoyl-CoA synthesis versus arachidonoyl-CoA synthesis favors the occurrence of two different acyl-CoA synthetase enzymes in rat brain microvessels. The time course of heat inactivation of arachidonoyl-CoA synthetase in rat brain microvessels is comparable to that reported by Reddy and Bazan (1983) with rat brain microsomes. Indeed, complete purification and characterization of the proteins responsible for these metabolic reactions will provide full evidence of two different enzymes.

Our data resemble those published by Wilson et al. (1982) and Neufeld et al. (1984*b*), who first discovered and analyzed an arachidonoyl-CoA synthetase in human platelets. Further, specific arachidonic acid CoA activation has been demonstrated in rat brain microsomes (Reddy and Bazan, 1983; Reddy et al., 1984); in human, bovine, rat, and frog retina (Reddy and Bazan, 1984); and in murine thymoma EL4 and cytotoxic T lymphocyte clones (Taylor et al., 1985). Neufeld et al. (1984*a*) selected a mutant $HSDM_1C_1$ fibrosarcoma line lacking an arachidonate-specific acyl-CoA synthetase which might account for arachidonic acid uptake. These data must be related together with the role of this particular fatty acid as a precursor of prostaglandins and leukotrienes in arterial wall cells and microvessel cells. Interestingly, Spector et al. (1983) suggested that thrombin-stimulated production of prostacyclin by arachidonic acid-incubated human vein endothelial cells was due to a larger amount of that fatty acid in the intracellular lipid substrate pools. Hence, as shown in this work, purified rat brain capillaries also exhibit a rather highly specific acyl-CoA synthetase activity toward arachidonic acid. Again, it raises the question of whether the activity of this ligase is involved in regulating the production of prostaglandins and leukotrienes. This is of course very relevant to the physiology of brain microvessels capable of producing prostacyclin (Baba et al., 1985) which may regulate cerebrovascular pressure (Chapleau and White, 1979; Pickard et al., 1980) as well as unknown functions of that particular structure.

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